Development of Mosquitocidal Herbal Nanoemulsions for Controlling the Dengue Vector, *Aedes aegypti*

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INTRODUCTION

Mosquitoes are hematophagous vectors as they transmit many diseases like malaria, dengue, filaria, chikungunya, Japanese encephalitis, etc. in humans. Dengue is an emerging viral disease, caused by dengue virus (DENV) and transmitted through female *Aedes aegypti* (Linnaeus). Dengue is pervasive throughout the tropic and sub-tropic areas, with some influencing spatial variation like humidity, rainfall, temperature and urbanization. According to the report of World Health Organization (2017) incidence of dengue, estimates that 3.9 billion people in 128 countries including India, are at risk of infection with dengue virus. In India according to the report of National Vector Borne Disease Control Program (NVBDCP, 2017) during 2017, 188401 cases and 325 deaths have been reported.

Vector borne diseases can be controlled if the disease transmitting vectors are controlled or prevented from biting humans. Plenty of research work has been done to control mosquitoes. In many countries, chemical and biological methods are used to control mosquitoes but they are not successful. This may be due to the fact that exposure of mosquitoes with synthetic chemical insecticides results in the development of resistant strain moreover, they are not considered safer for the environment.

This has attracted the attention of researchers towards the search for plant based extracts in controlling mosquitoes and extensive research has been conducted in the last decade. Moreover, essential oils from plants are used since a long period of time for repelling insect and now they are used to develop formulations which are supposed to be more effective and long lasting. Essential oils like lemon, *Eucalyptus* and citronella oils have been registered as common insect repellents in United State Environmental Protection Agency (Lee, 2018).

Since, the advent of nanotechnology, this multidisciplinary approach has grabbed the attention of researchers for developing insecticides. Metallic nanoparticles were synthesized using plant bio-reduction processes and screened for their mosquitocidal activity. However, there are nanotoxicity concerns for using metallic nanoparticles. Therefore, nanoemulsions are gaining

attention due to their biodegradable nature and plant origin. Recently, synthesis of oil in water based nanoemulsions formulated by dispersion of immiscible liquids with stabilizer or emulsifiers are becoming popular for various bio activities and applications like they have good pediculicidal activity (Lucia *et al.,* 2017), aphid repellent activity (Pascual - Villalobos *et al.,* 2017) and anti-bacterial activity (Nirmal *et al.,* 2018). There are few reports, where mosquito larvicidal activity of nanoemulsions has been documented (Nuchuchua *et al.,* 2009; Sakulku *et al.,* 2009; Anjali *et al.,* 2012; Montefuscoli *et al.,* 2014 & Sugumar *et al.,* 2014). However, trace studies are done to enhance bioactivity of nanoemulsion through combinatorial approach (Reegan *et al.,* 2014). Therefore, present research is planned to develop and characterize herbal nanoemulsions using combination of essential oils and evaluation of their mosquitocidal activity for controlling the dengue vector, *Aedes aegypti*.

REVIEW OF LITERATURE

Mosquitoes are widespread throughout the world. Also, they are the well-known vectors for transmitting various diseases. They can be controlled either by killing them or by preventing their bites. Various conventional and non-conventional methods have been used to control mosquitoes including chemical and plant based formulations.

Use of plants and their products is becoming popular for the development of insecticides as conventional insecticides are causing resistance problem. Essential oils are also gaining attention of researchers due to their countless properties in many fields. Amer and Mehlhorn (2006) studied larvicidal properties of lemongrass, *Eucalyptus* and camphor oils against *Aedes*, *Culex*, and *Anopheles* larvae. Prabuseenivasan *et al.* (2006) reported anti-bacterial activity of several essential oils including *Eucalyptus*, lemongrass and camphor. Tzortzakis and Economakis (2007) reported antifungal activity of lemongrass essential oil while Leimann *et al.* (2009) found its antimicrobial activity. Also, Cheng *et al.* (2009) reported larvicidal effect of *Eucalyptus* oil whereas Turgis *et al.* (2009) reported the anti-microbial activity of mustard oil. These essential oils possessed promising mosquitocidal properties and were further explored for their nanotechnological applications in the field of vector control.

In the last decade extensive research was carried out to explore mosquitocidal activity of green synthesized silver nanoparticle however, there are major toxicity concerns for their application in aquatic ecosystem. Bar-Ilan *et al.* (2009) conducted toxicity assessments of multisized gold and silver nanoparticles in zebrafish embryos and reported their LC_{50} values. Green synthesis of nano insecticides uses biological compounds which are eco-friendly and are safer than the chemically synthesized compounds. Nanoemulsions are gaining further attention of scientist in the field of vector control. Sakulku *et al.* (2009) have characterized and tested mosquito repellent activity of citronella oil nanoemulsion and found that high pressure homogenization process, surfactant and glycerol concentration have influenced release kinetics and mosquito protection time. Nuchuchua *et al.* (2009) studied *in vitro* characterization and mosquito (*Aedes*

aegypti) repellent activity of essential oils loaded nanoemulsions. The result suggested that the combination could improve the physical stability and prolong protection time. Anjali *et al.* (2012) synthesized stable neem oil nanoemulsion and checked its efficacy against *Culex quinquefasciatus* larva. They found that neem oil nanoemulsion was potent to act as a larvicide at LC_{50} 11.75 mg/l of concentration.

Montefuscoli *et al.* (2014) designed and developed aqueous nanoformulations for mosquito control. Their results indicated that the nanoemulsions are novel and can be used for pest management along with mosquito control. Sugumar *et al*. (2014) formulated nanoemulsion of *Eucalyptus* oil and tested its larvicidal activity against *Culex quinquefasciatus* the results indicated that nanoemulsion formed by using *Eucalyptus* oil exhibits natural pesticide activity. Pant *et al.* (2014) studied insecticidal activity of *Eucalyptus* oil nanoemulsion with *Karanja* and *Jatropha* aqueous filtrates and proved that the formulated nanoemulsion was potent to act as an insecticide and effective for longer period of time. Reegan *et al.* (2014) studied synergistic effect of essential oil based cream formulation containing five oils namely *Ocimum*, lemongrass, citronella, camphor and orange against *Culex quinquefasciatus* and *Aedes aegypti*. Owokotomo *et al.* (2015) studied in-vitro anti-cholinesterase activity of essential oil from four tropical medicinal plants indicating different degrees of AChEI potency for the selected essential oil extracts. Oliveria *et al.* (2016) developed a larvicidal nanoemulsion against *Aedes aegypti* with *Pterodon emarginatus* vogel oils and studied enzymatic assay on *Aedes aegypti* larvae and non-target organism.

Hazra *et al.* (2017) reviewed the methods of mosquitocidal formulation and stated that botanical and herbal methods are effective and eco-friendly for protection against the vector mosquitoes. Ramar *et al.* (2017) investigated nano insecticidal formulations from essential oil (*Ocimum sanctum*) also the knock down time and insecticidal activities of *Culex quinquefasciatus* and *Aedes aegypti* was recorded.

Recently, Mishra *et al.* (2018) formulated biological nano pesticides as a greener approach towards the control of mosquito vectors and also studied their effect on non-target species. Tavarrs *et al.* (2018) reviewed various synthetic and natural insect repellent formulations. While, Ricaurte *et al.* (2018) studied physical, thermal and thermodynamical properties of high oleic palm nanoemulsions.

It is clear from the literature survey that essential oils have been screened for their larvicidal and repellent activity. However, reports on their nanoemulsion development and mosquitocidal activity are less documented, especially with our selected oils namely *Eucalyptus*, lemongrass, camphor and mustard. Moreover, there is no report on combinations of these selected oils. The methodologies for nanoemulsion development are also different. A report on *Eucalyptus* oil nanoemulsion is known which shows promising effect against mosquitoes and we will be using a combinational approach for enhancing its bioactivity. Therefore, in the present study, oil in water based herbal nanoemulsions of these selected oils and their combinations would be developed and characterized to observe their mosquitocidal effect against the dengue vector, *Aedes aegypti*. Additionally, any potential neurotoxicity would also be evaluated.

OBJECTIVES

- Development and characterization of herbal nanoemulsions and their combinations.
- Bio-efficacy of synthesized herbal nanoemulsions against larvae of *Aedes aegypti.*
- Evaluation of repellency of herbal nanoemulsions against adult *Aedes aegypti*.
- Evaluation of potential neurotoxicity of herbal nanoemulsions against *Aedes aegypti*.

MATERIALS AND MEHTODS

MATERIALS

1. **Experimental animal: *Aedes aegypti* (Linnaeus, 1762)**

CLASSIFICATION	
Kingdom	Animalia
Class	Insecta
Order	Diptera
Family	Culicidae
Genus	*Aedes*
Species	*aegypti*

Commonly called as yellow fever mosquito, found in most parts of the world. It is an ectoparasite which is a vector of dengue, yellow fever, chikungunya, etc.

Habitat: *Aedes aegypti* mostly breeds in fresh water and found in peridomestic areas such as coolers, tanks, pots, etc.

Life cycle: Life cycle of *A. aegypti* comprises of four stages, three of which are aquatic while fourth is aerial. These stages are as follows:

(a) **Egg:** Female *A. aegypti* lays eggs in water and eggs hatch within 2-3 days.

(b) **Larva:** Legs are absent but respiratory siphon is present. The larva is found below the surface of water making an angle of 45° and swims by performing S shaped wriggling movement. They are voracious feeders. Larva develops through four stages or instars and after the IV instar they metamorphose into pupa.

(c) **Pupa:** The pupa is a non-feeding stage and it looks like a comma.

(d) **Adult:** The body has three distinct regions- head, thorax and abdomen. *A. aegypti* can be easily differentiated from other mosquitoes as it has white strips on legs and few other body parts including two lateral white bands on head.

2. **Essential oils:** Essential oils are generally extracted from plant parts by steam distillation process. They are hydrophobic in nature and have many applications due to their enormous properties. In our study camphor (*Cinnamomum camphora*), eucalyptus (*Eucalyptus globulus*), lemongrass (*Cymbopogon citratus*) and mustard (*Brassica campestris*) are selected based on their properties briefly described here:

   Camphor is used as a traditional medicine. It is used as a culinary spice, common insect repellent and flea killing substance (Hirota and Hiroi, 1967). Also, *Eucalyptus* oil has many biological properties like antimicrobial, herbicidal, fungicidal, insecticidal, nematicidal and insect repellent properties (Batish *et al.*, 2008). Lemongrass mostly grows in north-east India and the oil has preservative and fungicidal properties. Mosquito repellent properties of lemongrass have also been observed (Oyedele *et al.*, 2002). Whereas, mustard oil was used as a base for repellents which imparts longer protection time as compared to coconut oil against *Aedes albopictus* (Das *et al.*, 2003). Selected oils will be procured from authorized dealers with certificate of analysis.

METHODS

1. **Collection of test organism:**

   Larvae of *Aedes aegypti* will be collected from natural ovipositioning sites in Agra region, India. They will be maintained in laboratory at 27°±2°C temperature for performing larvicidal bioassay. Also, adults will be maintained for repellency tests. Adults will be provided with 10% sucrose solution. The adult mosquitoes will be starved for two to three days before the initiation of each repellency test. Standard procedure will be followed for the maintenance of larvae and adults in the laboratory (Govindarajan, 2011).

2. **GC-MS Analysis of selected oils:**

GC-MS analysis of all the four selected oils will be done for the analysis of chemoprofiling (Sugumar *et al.*, 2014).

3. Synthesis of Nanoemulsions:

Nanoemulsions will be synthesized by using ultrasonication method (Sugumar *et al.*, 2014). Essential oils will be used for the synthesis of these nanoemulsions, along with tween 80 (Polyoxyethylene (20) sorbitan monooleate) which will act as an emulsifier because it is hydrophilic and non-toxic. For all the preparation purified water will be used. According to the protocol followed different ratio of oil and surfactant/emulsifier will be taken for their preparation *viz.* 1:0.25, 1:0.5, 1:1, 1:1.5 and 1:2. Single oils and their combinations which would be stable will be used for bioassays.

4. Characterization of synthesized nanoemulsion:

Thermodynamical study will be evaluated for determining the stability of synthesized nanoemulsions (Shafiq *et al.*, 2007). Polydispersity index, zeta potential and droplet size of nanoemulsions will be taken by using Dynamic Light Scattering (Malvern Zeta Sizer) from Department of Chemistry, D.E.I., Agra for finding the stability of nanoemulsions and TEM will be performed at JNU, New Delhi for determining the droplet shape and size of the nanoemulsions.

5. Larvicidal bioassay:

The larvicidal activity of synthesized Nanoemulsions and their combinations will be checked against III instars of *Aedes aegypti* according to the WHO (2005) protocol. Positive and negative control will be setup with each test with permethrin and deionized water respectively. Experiment will be done in replicates. Mortality will be recorded after 24 hr and 48 hr in each case. Larvicidal bioassays that demonstrate more than 20% mortality in control will be discarded and repeated again. If less than 20% mortality will be observed, it will be corrected by using Abbott's formula (Abbott, 1925).

Abbott's formula:

C%M = (T – C) / (100 – C) *100

C%M = Corrected % Mortality

T = % Mortality in treatment

C = % Mortality in control

6. Repellent activity:

Repellent activity of nanoemulsions will be tested according to standard repellency protocol of ICMR, NVBCPD and NCDC (2014). The experiments will be carried out in the net cage (40×40×40) cm^3, containing 100 female *A. aegypti*, pre-starved for 12 hr. Tests will be conducted on five plastic cups with 10% sugar solution soaked on cotton. The cups will be treated separately with different range of concentrations of nanoemulsion formulation. DEET (N,N-diethyl-meta-toluamide or N,N-diethyl-3-methylbenzamide) will be used as positive control and only sugar solution soaked cotton will be used as negative control. The four treated cups will be placed in four opposing corners while the untreated negative control in the middle of the cage floor. Three minute landing counts will be made at an interval of 30 minutes. The experiment will be conducted in replicates with a series of test concentration. The % repellency will be calculated by: **% Repellency = $[(T_a - T_b)/T_a]$ x 100%** where,

$\mathbf{T_a}$ = number of mosquito landing received in control group and $\mathbf{T_b}$ = number of mosquito landing received in treated group.

7. Neurotoxicity assay:

For the evaluation of any potential neurotoxic effect of nanoemulsions, five III instar larvae of *Aedes aegypti* will be taken from control and test group in chilled phosphate buffer (0.1 M sodium phosphate, pH 7.4) for preparing homogenate. The homogenate will be centrifuged at 8000 rpm at 4°C for 10 minutes (Sugumar *et al.*, 2014). The supernatant will be collected and used as the enzyme source. This will be followed by its quantification, using Bradford method (Bradford, 1976; Kurger, 2009). According to the standard procedure, known quantity of bovine serum albumin will be used as standard with different range of concentrations. Amount of unknown protein will be

taken with Bradford reagent and incubated for 5 minutes at room temperature. The absorbance will be recorded at 595 nm for both. Acetyl cholinesterase activity will be tested by using Ellman's method (Ellman *et al.*, 1961). Prepared enzymes will be incubated with inhibitors 10 min before the addition of 5,5'-dithiobis-(2-nitobenzoic acid) (DTNB) and acetylthiocholine iodide of concentration 0.3 mM and 0.4 mM respectively. The final mixture will be incubated for 30 min at room temperature. The absorbance will be taken at 412 nm.

8. Statistical analysis:

LC_{50} and LC_{90} will be calculated by Probit analysis (Finney, 1971) and values will be expressed as mean ± standard deviation (SD). Statistical significance of differences will be examined using student t-test or analysis of variance (ANOVA).

PILOT STUDY

- Extensive literature survey has been done.
- Pilot study was conducted for selection of nanoemulsion and their larvicidal efficacy screening was also done.
- Four oils namely camphor (*Cinnamomum camphora*), eucalyptus (*Eucalyptus globulus*), lemongrass (*Cymbopogon citratus*) and mustard (*Brassica campestris*) were selected on the basis of pilot study.
- Nanoemulsion synthesis protocols were optimized.

PLAN OF WORK

1. **Collection of test organism (*Aedes aegypti*)**

    - Collection of *Aedes aegypti* larvae from its natural ovipositioning sites from Agra region and their maintenance in laboratory (Govindarajan, 2011).

2. **Development of herbal nanoemulsions**

    - Tween 80, essential oil and purified water will be mixed in different ratio and subjected to ultra-sonication (Sugumar *et al*., 2014).

3. **Charcterization of herbal nanoemulsions**

    - Thermodynamical studies for stability analysis (Shafiq *et al.*, 2007).
    - DLS (Zeta potential, PDI) and TEM for stability and size analysis.

4. **Bioefficacy of herbal nanoemulsion**

    - Larvicidal bioassay against III instars of *Aedes aegypti* (WHO, 2005).
    - Repellency against adults of *Aedes aegypti* (ICMR, NVBDCP & NCDC, 2014).

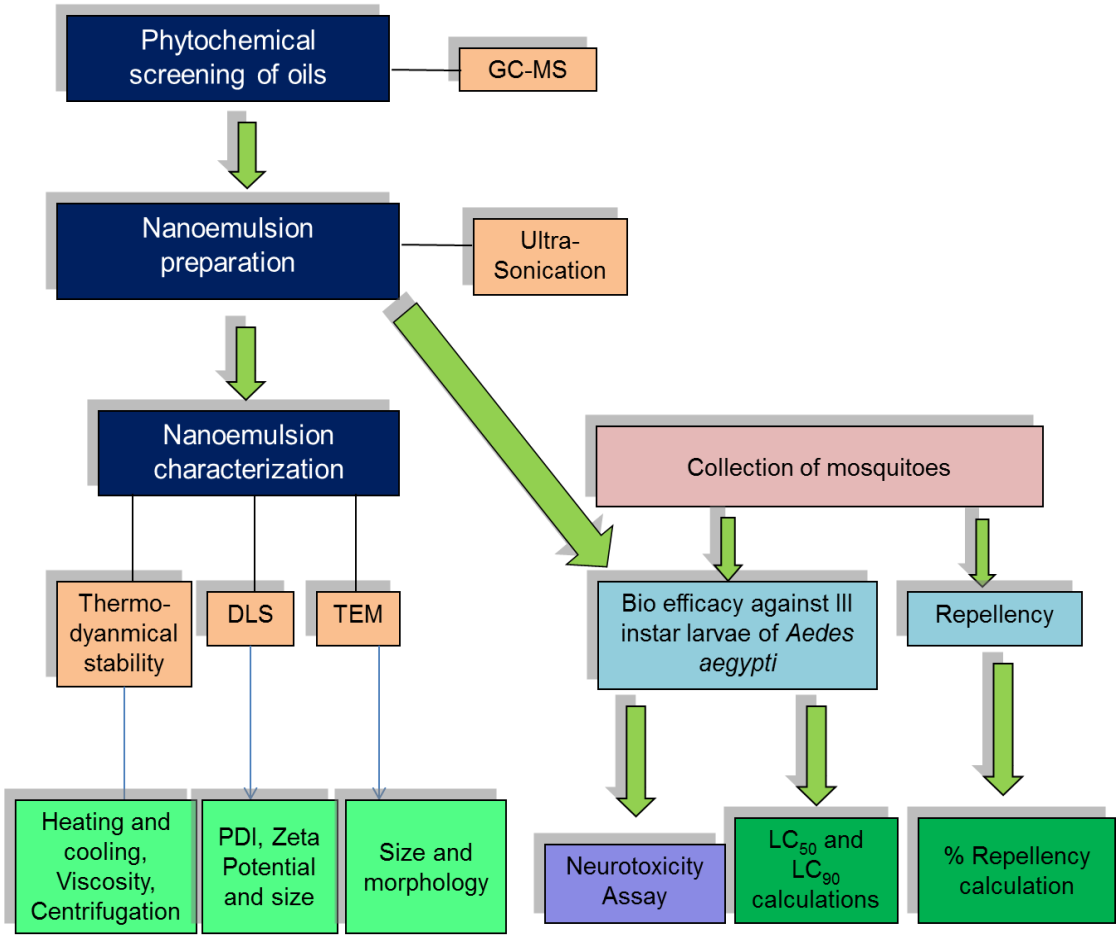
5. **Neurotoxicity assay**

    - Protein extraction and homogenate preparation (Sugumar *et al*., 2014).
    - Protein quantification (Bradford, 1976).
    - Ellman's assay (Ellman *et al.*, 1961).

6. **Probit and statistical analysis**

    - Calculation of LC_{50} , LC_{90} and % repellency (Finney, 1971).
    - Mean ± standard deviation (SD) and student t-test or ANOVA for statistical analysis.

EXPERIMENTAL DESIGN



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